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# The Role of the Dosage Compensation Complex as a Pathway for Spiroplasma to Induce Male Lethality in Drosophila melanogaster

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# The Role of the Dosage Compensation Complex as a Pathway for *Spiroplasma* to Induce Male Lethality in *Drosophila melanogaster*

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## Abstract

*Drosophila melanogaster* and many other insects harbor intracellular bacterial symbionts that are transmitted vertically from infected host mothers to their offspring. Many of these bacteria alter host reproductive developmental processes in order to increase their transmission success. For example, *Spiroplasma*, a spirochete that naturally infects *D. melanogaster*, selectively kills males during mid-embryogenesis while sparing females. Previous studies suggested that *Spiroplasma* interacts genetically with the male-specific dosage compensation pathway, which causes ~2-fold up-regulation of most genes located on the male's single X chromosome so that their expression matches the levels found in females who have two Xs. To further test this idea, I used confocal microscopy to visualize dosage compensation complex (DCC) localization and activity in infected as well as uninfected embryos. In the presence of *Spiroplasma*, the DCC became abnormally mis-localized across the nucleus. This pattern was accompanied by abnormal acetylation of histone H4K16, a mark induced by DCC activity and needed for proper X chromatin remodeling. My results imply that *Spiroplasma* directly targets the DCC by misdirecting it to uncompensated regions of the genome, an effect that leads to abnormal gene mis-regulation and consequent lethality (work from other members in our group). To further investigate this interaction, we transgenically expressed low levels of MSL-2 in both *Spiroplasma* infected and uninfected embryos in order to cause ectopic formation of the DCC in the female sex. I found that when infected, female embryos expressing the DCC showed significantly reduced viability in comparison to uninfected transgenic females. This result supports the notion that *Spiroplasma* uses the DCC in a dominant gain-of-function manner to kill embryos.

## Introduction and Background

Symbiosis is common within the eukaryotes. Although some cases of symbiosis are beneficial to the hosts, others are harmful. A number of bacteria that must live inside host cells and tissues can manipulate host reproduction in order to benefit their spread, at the cost of the host. A striking example is the killing of male host progeny by *Spiroplasma* bacteria in the arthropods. Several previous studies in flies provided clues to how males are targeted for death, but so much about this targeting is not understood at the molecular level. In this thesis, I have addressed how *Spiroplasma* targets the dosage compensation pathway to kill *D. melanogaster* males. I will provide some background on bacterial reproductive manipulators and then focus more on *Spiroplasma*, the dosage compensation pathway in *D. melanogaster*, and what is know about male-killing in this species. I will then present the results of my study. In short, I have found strong evidence that *Spiroplasma* directly targets the dosage compensation machinery to kill males and these findings have important implications for host-pathogen interactions.

### *Pathogens can manipulate host reproductive processes in order to increase their own transmission.*

A particular type of symbiotic relationship that benefits symbiont reproduction is reproductive parasitism, where females are selected for to continue the transmission of the symbiont and males are killed due to their role as dead ends (Hurst and Frost, 2015). In such cases, the bacterial symbionts are transmitted from mother to her progeny through the egg cytoplasm. In contrast, such bacterial symbionts are not transmitted through the sperm to new progeny presumably because the sperm has very little cytoplasm for the symbionts to exist within. Because of the symbionts' maternal transmission, males are

'dead ends' for transmission. Therefore, there are many cases in which the symbionts have evolved different ways of manipulating host reproduction to benefit the bacterial transmission to the next generation of progeny. One such example is conditional male sterility caused by bacteria known as *Wolbachia* that infect thousands of different insect species (Bourtzis and Miller, 2003). This conditional male sterility is known as cytoplasmic incompatibility (CI). In general, *Wolbachia* somehow marks the sperm's hereditary material so that if an infected male mates with an uninfected female, her progeny will die almost 100% of the time. However, if the female is infected and her eggs thus also have *Wolbachia* in the cytoplasm, then the presence of *Wolbachia* there causes the embryo to live – this is called rescue. If uninfected males mate with *Wolbachia* infected females, the progeny also survive. And, of course, uninfected males can produce viable progeny when mated with uninfected females. Thus, there is a strong selection against uninfected females, or in other words, a strong selection in favor of infected females. This action benefits the symbiont *Wolbachia* because more and more infected females will appear in the fly population.

*Wolbachia*-caused CI is perhaps the most well-known case of bacterial manipulation of host reproduction. However, several other types of manipulation exist. For example, in some arthropods such as wood lice, certain *Wolbachia* strains can morphologically convert genetic males into females that can then transmit the bacteria (Bourtzis and Miller, 2003). In other insects, specific bacteria can parthenogenesis, in which an infected female can produce viable and fertile offspring without the need for fertilization. Finally, some bacterial symbionts can cause the early death of male embryos, while sparing females. This syndrome, called male-killing, is thought to help

the spread of the bacteria because males are not as essential for transmission as females. By killing males, there are more limited resources such as food stores for the infected females, who can then spread more bacteria. These male-killing bacteria usually kill most but not all males so that a few exist for fertilization and health of the population.

Male-killing has not been explored enough yet to understand the molecular basis of this phenomenon. A specific question would be how, at the mechanistic level, the bacterial symbionts can selectively kill the male sex while sparing females when both sexes are infected with the symbionts. It is also important to eventually identify the specific means that the bacteria use in order to kill males. Such questions are important to address because they apply to not only a single insect species but many species since male-killing has been observed in a number of different of arthropods such as *Drosophila melanogaster*, *Adalia bipunctata*, *Acraea encedon*, and *Homona magnanima,* to name a few (Morimoto et al., 2001). In my thesis, I focus on the fruit fly *D. melanogaster* and a symbiotic male-killing bacterium called *Spiroplasma*.

***A specific strain of Spiroplasma kills males with an almost 100% efficiency.***

*Spiroplasma* is a symbiont that lives in certain numerous species of arthropods (Haselkorn, 2010). Not all strains of *Spiroplasma* are reproductive parasites, but I am interested in the male-killing *Spiroplasma* strain MSRO (for *melanogaster* sex ratio organism) that lives naturally within populations of *D. melanogaster*. Previous studies have shed some light on the interactions between MSRO (from here onward referred to simply as *Spiroplasma*) and its host *D. melanogaster*. In this host, *Spiroplasma* are primarily found within the hemolymph (an insect's version of 'blood') but they are also

present in the egg cytoplasm. During development, the female develops ovaries during the larval stages. At this time, the *Spiroplasma* in the hemolymph are capable of moving actively across tissue boundaries. They move from the hemolymph into the developing eggs where they will replicate and be present during fertilization (Haselkorn, 2010). Apparently *Spiroplasma* are very good at infecting developing eggs because nearly all eggs laid by infected females are infected with *Spiroplasma*.

Until fairly recently it was not known exactly when and how male progeny die due to the male-killing effect. Students from our lab, Trisha Chong and Jennifer Martin, addressed these issues by examining very young *Spiroplasma* infected males with confocal microscopy. They discovered a few important findings. Specifically, that males die during mid-embryogenesis as certain tissue types are becoming established. Trisha and Jennifer used antibodies as markers to highlight specific tissues during mid-embryogenesis. They found that most tissue types looked fine; however, as the central nervous tissues began to form, these tissues looked very abnormal in their morphology. The cells in the central nervous tissue looked abnormally spaced, compared to a very regular, ordered pattern in uninfected embryos. The axons of neurons also looked abnormal and they did not appear to migrate out into the developing lateral muscle tissues as they would normally do. These findings allowed our group to pinpoint the first window of time that the male-killing effect seems to occur during. We still do not know exactly why the neural tissues are first affected; although, my work may help to shed light on this issue (see discussion for details). But more importantly, this finding allows us to focus on understanding the cellular and molecular aspects involved in male-killing that presumably happen at the window of development before these morphological

defects occur. This finding, along with studies published from other groups, has allowed me to pursue the question of how exactly *Spiroplasma* accomplishes male-killing at the cellular level.

***The dosage compensation pathway as a possible target by male-killing Spiroplasma***

Researchers in the field of male-killing have assumed for a long time that whatever cellular process or molecule(s) is targeted by *Spiroplasma* must be specific in some way to the male sex. A couple of possible male-specific targets occur during very early male development. One of the first tested was the male's tissue-specific sexual identity. To test this characteristic as a possible bacterial target, researchers asked the question of whether mutations in several genes, including double-sex, were able to influence male-killing. In one experiment, researchers used double-sex mutations to convert infected males into individuals with female somatic tissue identity (Rédei, 1998). They found that these feminized males still died due to male-killing. Genetistics also performed reciprocal experiments in which infected females were converted into a type of male individual. These individuals did not experience lethality. These findings suggest that it is not the sexual identity per se that allows *Spiroplasma* to target the male sex.

Another very obvious candidate pathway is the male-specific dosage compensation pathway. In animals with different sex chromosomes, some sort of dosage compensation must be used to adjust the level of gene expression for genes located on the equivalent of the X chromosome. This is so that the number of genes in males, who have one X chromosome, match levels found in females, who have two X chromosomes. This compensation is important because, not only do males and females have different

numbers of the X chromosome, but also the X chromosome has hundreds of genes important for both sexes while the Y chromosome typically has just a few (10-20) genes needed solely for male fertility and development (Charlesworth, 2001). The way mammals perform X gene dosage compensation is by transcriptionally silencing one of the two X chromosomes at random in each female cell during early development so that the gene dosage in females is decreased to match the levels found in males.

Dosage compensation in many insects, like *D. melanogaster*, occur in a very different way. In these organisms, a set of proteins known as the male-specific lethal proteins are produced during early embryogenesis in both males and females. In males, these proteins, MSL-1, MSL-2, MSL-3, MALELESS (MLE), and MALES ABSENT ON THE FIRST (MOF), and a structural RNA known as roX, form a complex called the dosage compensation complex (DCC) (Conrad and Akhtar, 2012). The DCC forms as the roX RNA is transcribed (the roX gene is actually located on the X chromosome) and spreads to nearby chromatin regions. Additionally, the DCC is thought to bind to X sequences by associating with regions called chromatin entry sites, which contain motifs called male recognition sequences and are more enriched on the X chromosome compared to the non-sex chromosomes (Meller et al., 1997). On the X chromosome, the DCC enzymatically acetylates the histone H4, one of the four major DNA packaging proteins in chromatin. This chemical mark is thought to lead to some sort of chromatin change that allows an easier access of transcriptional proteins to the genes on the X chromosome (Gelbart et al., 2009). Females do not produce MSL-2, which blocks the DCC from forming and preventing this chromatin remodeling and gene compensation from happening. Since the normal function of the DCC is very important for gene

expression specifically in males, any alterations to the DCC or perhaps downstream aspects such as histone acetylation could lead to negative effects in males and possible preferential death of this sex.

***Experimental evidence that Spiroplasma targets the DCC.***

There is evidence that the DCC is in fact involved in the lethality of *Spiroplasma* infected *D. melanogaster* males. In a previous study, researchers tested this idea by using *Spiroplasma* infected *D. melanogaster* stocks that contained mutations of each of the five genes encoding the proteins that make up the DCC. These researchers wanted to determine if these mutations, which caused loss of DCC formation and function, might influence male-killing. They found that in the presence of any of these mutations, male-killing was suppressed and the infected males survived until the time that they would normally die due to the mutations' effects, death at the third instar larvae stage as a result of the lack of gene dosage compensation (Veneti et al., 2005). The main conclusion from this work is that the presence of a functional and complete DCC is required for male lethality to occur, but the reason for this is still unclear.

***My experiments planned to address whether Spiroplasma interacts with the DCC to cause male lethality, and if so, how.***

In my thesis work I aim to better understand the cellular basis of male-killing by *Spiroplasma*. There are several different specific hypotheses for how *Spiroplasma* could influence male death through the dosage compensation pathway. Previous studies have demonstrated that dosage compensation is achieved through acetylation of H4K16 on the X chromosome of males (Gelbart et al., 2009). This acetylation is accomplished

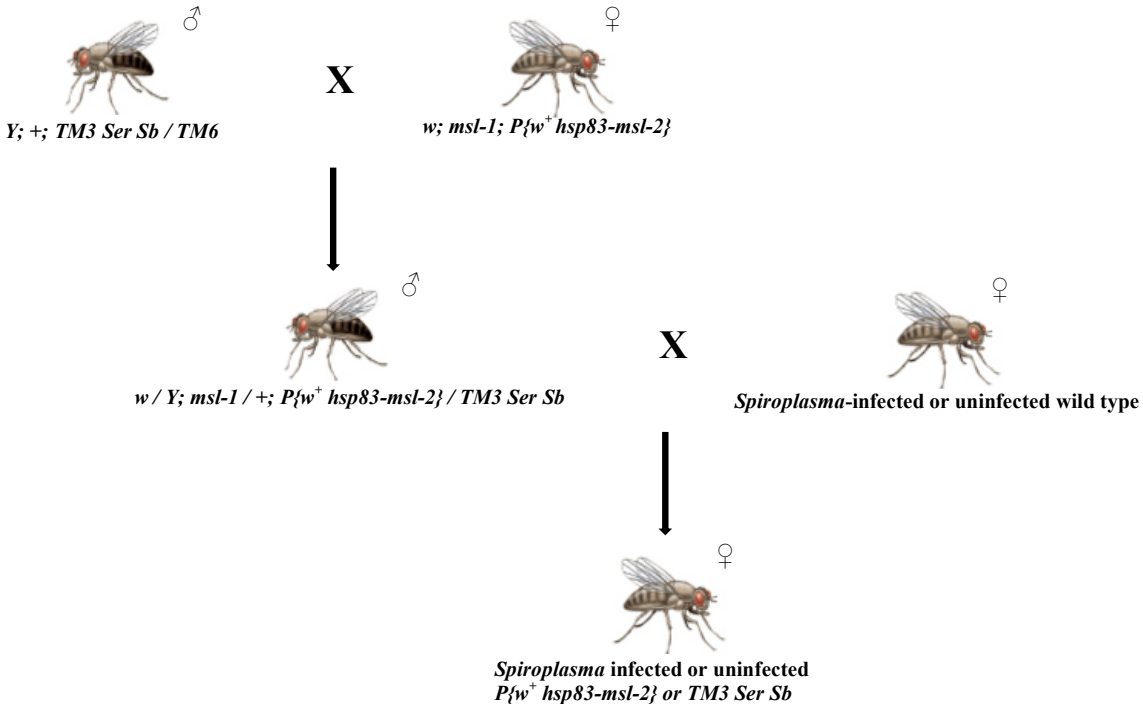
specifically by histone acetyltransferase MOF, an integral component of the DCC (Kind et al., 2008). One possible mechanism for male-killing is that *Spiroplasma* could cause the DCC to behave abnormally so that the acetylation activity of MOF is hyper or hypo active, leading to X chromosome gene mis-regulation and ultimately death of the organism. Another possibility is that *Spiroplasma* alters the ability of the DCC to properly bind to X sequences. Finally, it is also possible that *Spiroplasma* alters some unforeseen aspect of dosage compensation that is different from the above two possibilities in order to target males for death.

In this thesis I have tested these different hypotheses by directly visualizing the DCC as it forms during early embryogenesis, where it localizes within the genome, and its acetylation patterns both in the presence and absence of *Spiroplasma* in male embryos. My work strongly suggests that *Spiroplasma* kills male embryos by disrupting the ability of the DCC to properly bind only to the X chromosome, but instead, causing it to bind abnormally to non-sex chromosomes. I also present ideas for how this effect leads to male death and also some suggestions for how *Spiroplasma* may cause DCC mis-localization.

## Materials and Methods

### *Fly stocks and genetic crosses*

The *Spiroplasma* MSRO strain was obtained from a population of *D. melanogaster* flies caught in Uganda (Pool et al., 2006). In order to infect the specific fly lines used in this study, I used two different approaches. In one approach, I used a bacterial transfer method in which I removed some hemolymph (inter-tissue fluids) from the abdomen of very old (3 week old) infected females and injected them into the abdomens of very young (1-2 day old) females of a given genetic background. In this method, I was able to change only the infection status of the fly stock so that there would be no difference between the genetic backgrounds of the infected and uninfected fly lines. This made any experimental differences between these conditions attributable to the bacteria. Another way I obtained infected fly lines of a certain genotype was by crossing uninfected and infected flies together so that the bacteria was transmitted from mother to offspring in the given crossing scheme. I used this latter method in order to obtain the the infected MSL-2 transgenic line. Specifically, I crossed *w; msl-1; P{w$^+$hsp83-msl-2}* females (obtained from Mitzi Kuroda, Harvard University) to *w / Y; +; TM3 Ser Sb / TM6 Tub* males (Scheme 1). The resulting *w / Y; msl-1 / +; P{w$^+$hsp83-msl-2} / TM3 Ser Sb* male progeny were crossed to either *Spiroplasma* infected or uninfected *Canton-S* females. The resulting progeny were either carrying the MSL-2 transgene or the balancer chromosome. In this way I could obtain infected or uninfected embryos and artificially expressing MSL-2 or not adults.




**Scheme 1.** Genetic crosses for artificial induction of DCC in females.

### *Embryo Collection and Fixation*

Embryos were collected using empty plastic collection bottles, capped with grape-medium agarose plates, containing hundreds of crossed flies of the desired genotypes (Sullivan et al., 2001). The flies were allowed to lay eggs for several hours and then aged to the desired number of hours after egg deposit (AED) for accurately staged embryo collections. Collected embryos were placed into a mesh collection basket (screw cap microfuge tube with top and bottom sliced off with a razor blade) and washed vigorously with 50% bleach and a glass Pasteur pipette for 1 minute. They were then rinsed briefly with deionized water (DIW). The de-chorionated embryos were placed into a 10mL glass vial. Then, 2mL of heptane and 2mL of fixative were added into the vial. The fixative was made previously in a 10mL glass vial by combining 1.5mL 1xPBS and 500uL 16%

para-formaldehyde (PFA) to make 4% PFA. The 10mL vial with the embryos, heptane, and fixative was left on a platform rocker for exactly 8 minutes. After the 8 minutes, the fixative at the bottom of the vial was removed and 2mL of methanol was added to the vial. The vial was shaken as vigorously as possible for 15 seconds and the vial was placed onto the bench top in order to let the embryos settle to the vial's bottom. All of the heptane at the top was removed, along with any embryos that did not sink (these were not de-chorionated). Another 2mL aliquot of methanol was added, the vial shaken slightly to mix, and the embryos let to settle. This step was repeated a third time. The vial with embryos and methanol was labeled and placed in the -20$^{o}$C freezer for long term storage.

Hydration of embryos is necessary before antibody staining or FISH. In order to hydrate embryos, three 1mL aliquots of methanol:1xPBT dilutions were made: 9:1, 5:5, and 1:9. First, the embryos were transferred from the 10mL methanol storage vial into a 1.5mL centrifuge tube. All the methanol was removed and 1mL of the 9:1 dilution was added into the tube. The embryos and dilution mixture were pipetted up and down slowly to mix. Then, the embryos were allowed to settle to the bottom of the tube and the dilution was removed. The 5:5 and 1:9 dilutions were introduced to the embryos in a similar procedure. The embryos were then washed three times with 1mL of pure 1xPBT for 5 minutes each on the platform rocker. Finally, the embryos were transferred into a 0.6mL centrifuge tube with 1xPBT and used immediately for antibody staining.

***Antibody staining and fluorescence in situ hybridization (FISH) of fixed embryos***

Staining embryos with antibodies allowed me to visualize certain proteins of interest by using fluorescence confocal microscopy. Immuno-staining involves a primary

antibody that targets a specific protein and a fluorescently-labeled secondary antibody that specifically recognizes the primary antibody. The fluorescence tag is what enables the proteins of interest to be seen with the microscope.

The primary antibodies included mouse anti-GFP (4C9) from Developmental Studies Hybridoma Bank in a 1:50 dilution, goat anti-MOF (dn-17) from Santa Cruz Biotechnology at a 1:100 dilution, and rabbit anti-acetyl-histone H4 lysine 16 (07-329) from Millipore at a 1:100 dilution. The secondary antibodies included anti-mouse, anti-rabbit, and anti-goat labeled with either Alexa 555 or Alexa 633 from Molecular Probes-Invitrogen at a 1:300 dilution. The Y fluorescence in situ hybridization (FISH) probe contained 5′-AAT ACA ATA CAA TAC AAT ACA ATA CAA TAC-3′ and was synthesized by IDT. The X16E FISH probe was provided by C.T. Wu and D. Colognori (Harvard University). Each of the FISH probes were hydrated by adding 12uL UltraPure DIW to the stock and the probes were diluted by combining 2.5uL of the hydrated stock with 17.5uL UltraPure DIW. I explain the general procedure of FISH below, which normally follows antibody staining

Antibody staining involved removing the 1xPBT from the 0.6mL tube containing hydrated embryos and adding the diluted primary antibody. The tubes were allowed to incubate overnight on a rotisserie in the refrigerator. On the next day, the embryos were washed three times with 1xPBT for 10-15 minutes each time on the platform rocker. The 1xPBT was removed and the diluted secondary antibody was added into the tube. The embryos were allowed to incubated for 1 hour on the platform rocker while covered with aluminum foil to block any light. Finally, the embryos were washed with 1xPBT three times for 10-15 minutes each on the platform rocker while covered with the foil.

Following antibody staining, FISH was conducted on all embryos because I used the Y probe to distinguish female from male embryos. The stained embryos were re-fixed with 4% PFA for 1 hour on the platform rocker while covered in foil. The post-fixed embryos were then quickly washed three times using 1xPBT and then three times with 2xSSCT for 5 minutes each. After these washes, the embryos were washed for 10 minutes each using 20% formamide/SSCT, 40% formamide/SSCT, and 50% formamide/SSCT. The embryos were then placed in a 37$^{o}$C water bath for 1 hour in a fresh aliquot of 50% formamide/SSCT. After the 1-hour incubation, the embryos were moved into a PCR tube, the 50% formamide/SSCT was removed, and 1.5uL-2uL of the FISH probe and 36uL of hybridization buffer were added and mixed thoroughly in the tube. The tube was placed into the PCR machine and incubated at 92$^{o}$C for 2 minutes and then at 30$^{o}$C overnight. After overnight incubation, the embryos were washed with 50% formamide/SSCT three times for 10 minutes each, 40% formamide/SSCT one time for 10 minutes, and 20% formamide/SSCT one time for 10 minutes on the platform rocker while covered in foil. Finally, the embryos were washed with 2xSSCT three times for 5 minutes each and mounted on a slide with VectaShield, DNA stain DAPI, from Vector Labs Inc. A coverslip was placed on top of the sample, sealed with nail polish, and allowed to dry for at least 45 minutes in the dark before either imaging immediately or freezing for later imaging.

***Microscopic Imaging of Stained Embryos***

Imaging of embryos was performed on the Leica TCS SPE confocal microscope. All images were taken at the same laser power and gain, as well as processed with the

same changes on brightness and contrast so that the images between different genetic conditions (infected or uninfected) could be directly compared for differences in staining patterns. The images were processed on Adobe Photoshop CS5 version 12.

## Results

***Dosage compensation misbehaves in Spiroplasma infected males.***

In order to investigate if and how *Spiroplasma* alters dosage compensation, I visualized the DCC directly using immuno-cytology. To tell the difference between male and female embryos, I used a Y probe. The Y probe was only seen in male embryos and not in female embryos under the confocal microscope (Figure 1). This method allowed me to distinguish between males and females when obtaining data and making comparisons.

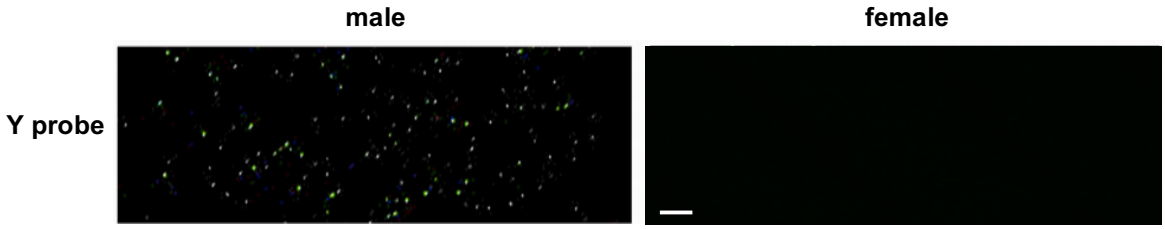



**Figure 1.** Y probe is present in male embryos, but not female embryos. Scale bar: 15um.

I was able to image the DCC by staining infected and uninfected embryos with an antibody directed against MOF, a major component of the DCC. I also stained these embryos with a H4K16 acetylation antibody because previous studies have named MOF as responsible for this important acetylation and resulting gene regulation.

Confocal imaging showed no DCC formation at 2-3 hours AED in uninfected control male embryos (Figure 2). This is expected because at this time, the DCC is just forming (Conrad and Akhtar, 2012). Similarly, there appeared to be no H4K16ac signal either as MOF and subsequently the DCC is needed to initiate acetylation. At 5-6 hours AED, strong and concentrated MOF as well as H4K16 acetylation signals are seen in control embryos. These signals became even more pronounced and abundant in control males 10-12 hours AED. It is also noteworthy to mention that MOF and H4K16ac perfectly co-localized with each other in the nucleus.

Looking at infected male embryos, I saw discrepancies in MOF and H4K16ac patterns in comparison to their control counterparts (Figure 2). These differences appeared later in embryogenesis as at 2-3 hours AED, there was not MOF and H4K16 present in the embryos ($n_{control} = 7$). This concurred with what was seen in the control embryos ($n_{infected} = 6$). This means that *Spiroplasma* does not make DCC form earlier than it naturally does. Major differences in the MOF and H4K16ac patterns between the control and infected manifested around 5-6 hours AED ($n_{control} = 13$; $n_{infected} = 13$). Both MOF and H4K16ac appeared more spread out across the nucleus of cells in the infected embryos. However, co-localization of MOF and H4K16ac was still present in these infected male embryos. This suggests that the DCC is possibly abnormally localizing at ectopic sites rather than concentrated on the X chromosome. Even more discrepancy in MOF and H4K16ac signals was seen 10-12 hours AED ($n_{control} = 11$; $n_{infected} = 15$). Both signals were much weaker in the infected embryos. This might be because DCC is being degraded as the embryo starts to die. The nuclei of infected male embryos during this stage of embryogenesis also appeared more hyper-condensed compared to nuclei in control male embryos of similar age. This leads to questions about whether gene expression is being affected by this unusual acetylation of chromatin and hyper-condensation of DNA in the nucleus.

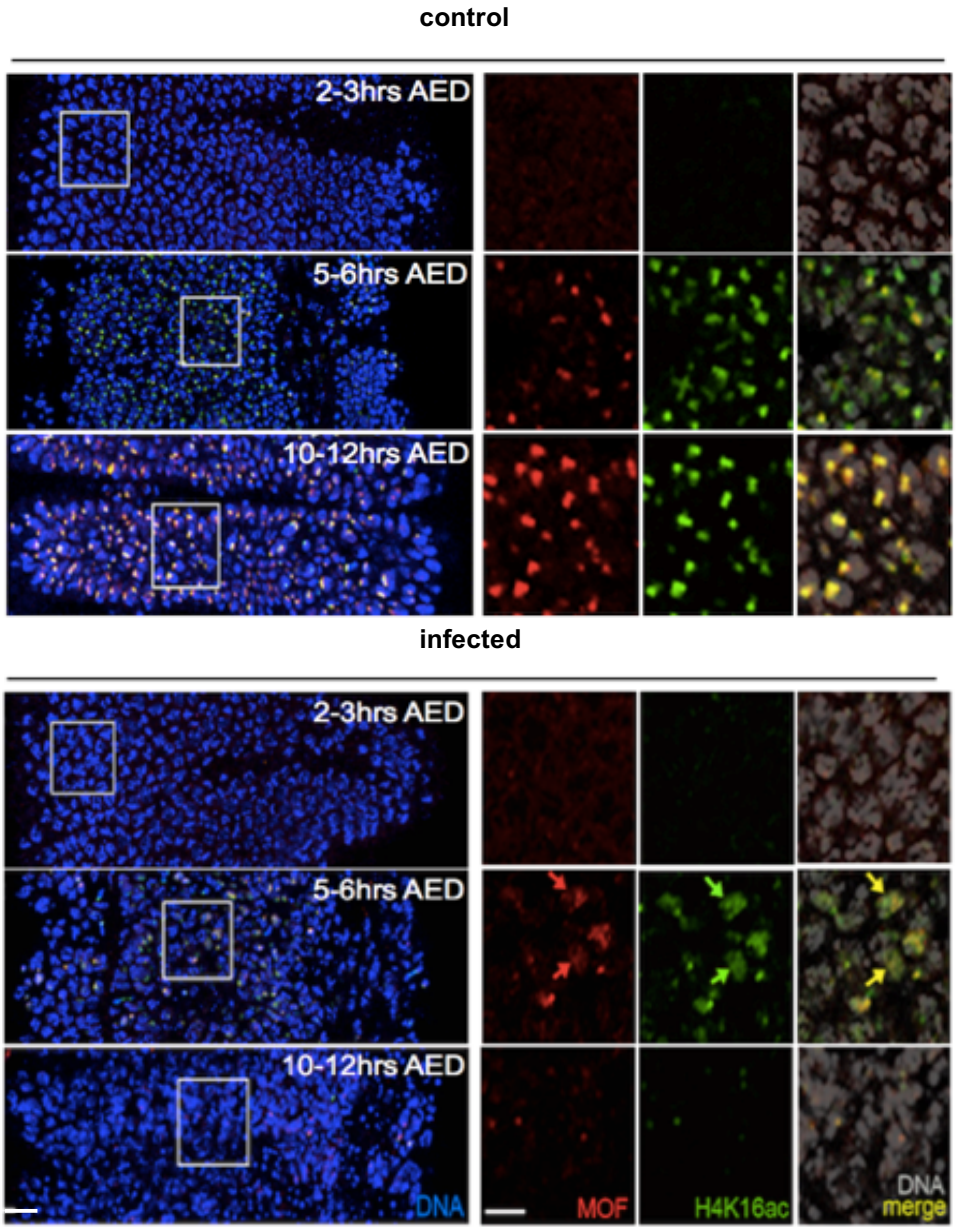



**Figure 2.** MOF and H4K16ac co-localize, but mis-localize across nucleus in infected males. Major discrepancies in MOF and H4K16ac signals first occur at 5-6 hours AED (arrows). Scale bar: 15um in the low-magnitude images in the left and 5um in the high-magnitude images on the right.

In order to address this question of gene expression, I imaged 10-12 hours AED male embryos stained with X16E, a marker for the X euchromatin, and H4K16ac. These two specific stainings are important because acetylation normally occurs on the X chromosome so that it can be dosage compensated in males as they only have one X chromosome. Control and infected male embryos both expressed X16E signals, which shows that *Spiroplamsa* is not affecting X euchromatin and rules out the possibility of *Spiroplasma* affecting how much X euchromatin is actually present in the infected males (Figure 3). Therefore, the problem is not a reduction of X euchromatin present in these males leading to gene mis-expression and lethality, but rather there is the possibility of a decrease in the availability of these genes to be transcribed being the problem.

Even though both control and infected males showed similar X16E patterns, control males had overall stronger H4K16ac signals. H4K16ac signals in infected males were weak and more scattered in the embryo. This is in agreement with H4K16ac patterns in the previous figure. Also, in infected males, X16E and H4K16ac signals were not closely associated with each other. Some X16E signals were neither directly next to more co-localized with H4K16 signals. In comparison, control male embryos showed close association between the two signals. A possible explanation for this observation of lower H4K16ac is that the DCC is being kicked off the X chromosome somehow. It is highly likely that this would have an impact on gene expression and thus, RNA sequencing was done by other members of our lab to determine this affect (see discussion for more details).

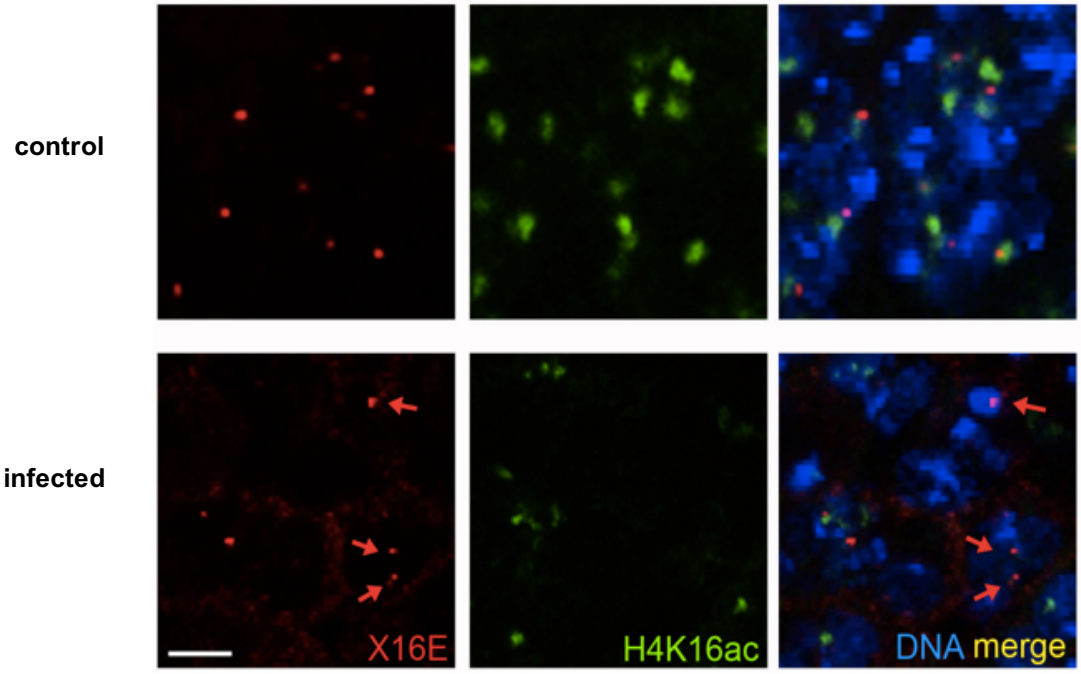



**Figure 3.** Association of X euchromatin and H4K16ac does not always occur in infected males. Some X regions do not have histone acetylation near them (arrows). Scale bar: 5um.

I also imaged control and infected females as insurance that the MOF and H4K16ac antibodies were staining correctly. As expected, both control and infected female embryos showed no MOF and H4K16ac signals (Figure 4) (n = 9). Any light faint signals appeared to be background staining when compared to MOF ad H4K16ac signals in control and infected males (Figure 2). This means that no abnormal DCC formation was occurring in females.

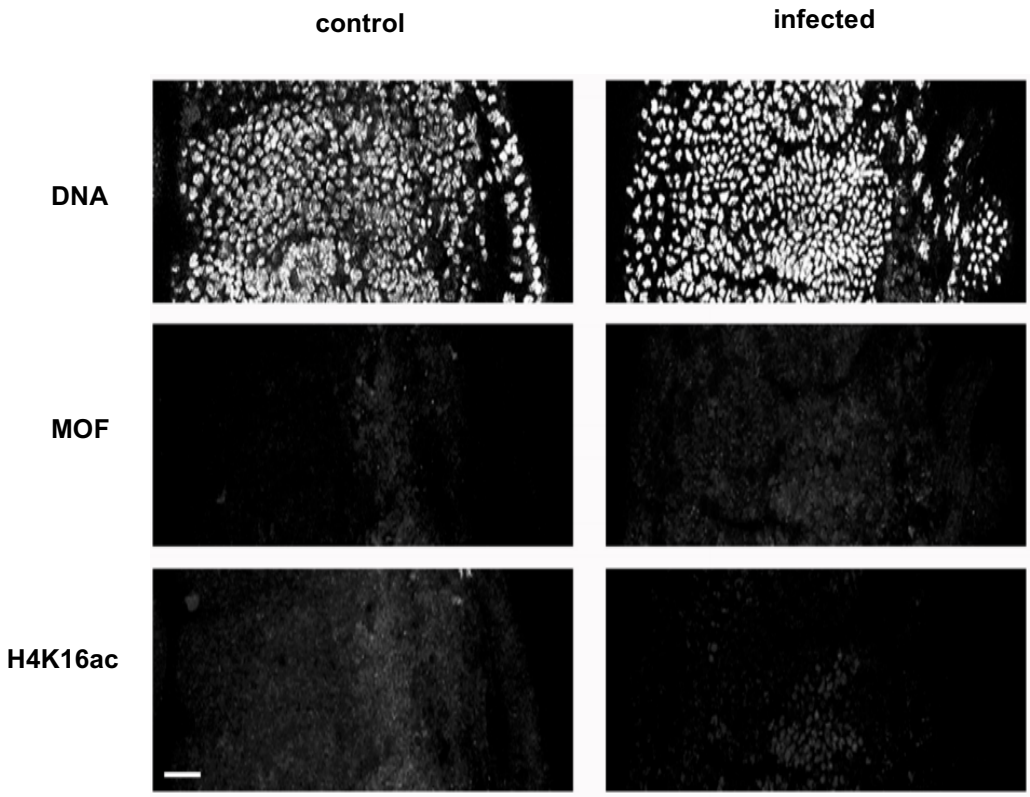



**Figure 4.** MOF and H4K16ac is not present in control and infected females. Scale bar: 15um.

***Spiroplasma infected females expressing the DCC experience lethality.***

To test whether *Spiroplasma* affected the DCC directly or indirectly through some other male-specific pathway, I looked at control and infected females that had a leaky MSL-2 transgene. This leaky transgene allows females to create some DCC while not inducing lethality in them, which is proved by the almost equal number of F1 transgenic and non-transgenic females (Figure 5). I also looked at lethality of these transgenic females and compared them to males with and without the same transgene. There was no large disparity between the number of F1 transgenic males and females and F1 non-transgenic males and females in the absence of *Spiroplasma* infection. However, there was a more than 2-fold decrease in F1 transgenic females in comparison to non-transgenic females in the presence of infection. In addition, both F1 transgenic and non-transgenic males showed a drastic decrease in survival (close to zero) which is expected because infected males with a complete DCC are almost guaranteed to die during embryogenesis. This means that female lethality is caused by an interaction between the DCC and *Spiroplasma* and thus, the DCC is being targeted by the bacteria.

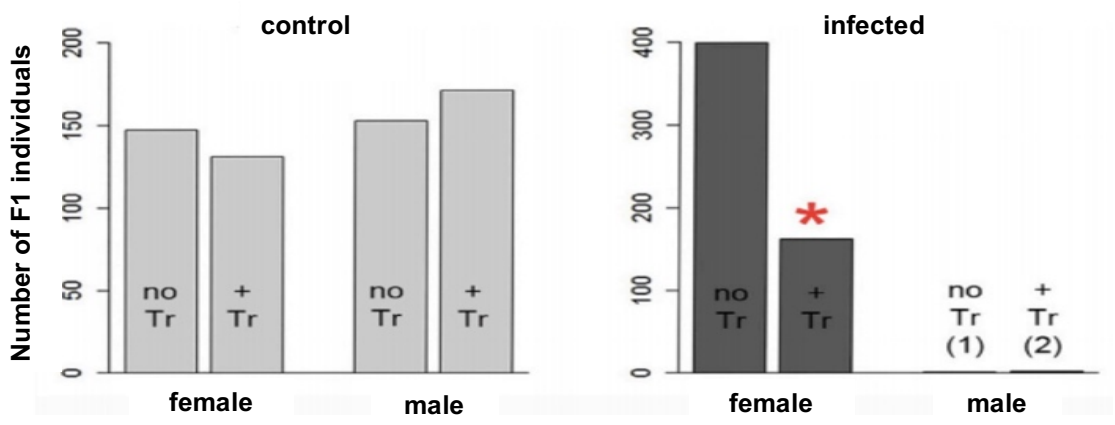



**Figure 5.** Transgenic females exhibit lethality in the presence of *Spiroplasma*.

I also used immuno-FISH and confocal microscopy to visualize the DCC in females by staining for MOF. 10-12 hours AED transgenic males and females showed similar decreases in MOF signal when comparing control and infected embryos (Figure 5). Control transgenic males exhibited stronger signals than control transgenic females, which was also the case in the infected embryos of both sexes. This is expected because transgenic females only form a little DCC. MOF signals were also very spread out in both infected sexes, which means that abnormal DCC localization is also occurring in transgenic females as it does in infected males. I can infer from this that *Spiroplasma* is affecting these females in the same way that it affects males and that this interaction is directly related to the DCC.

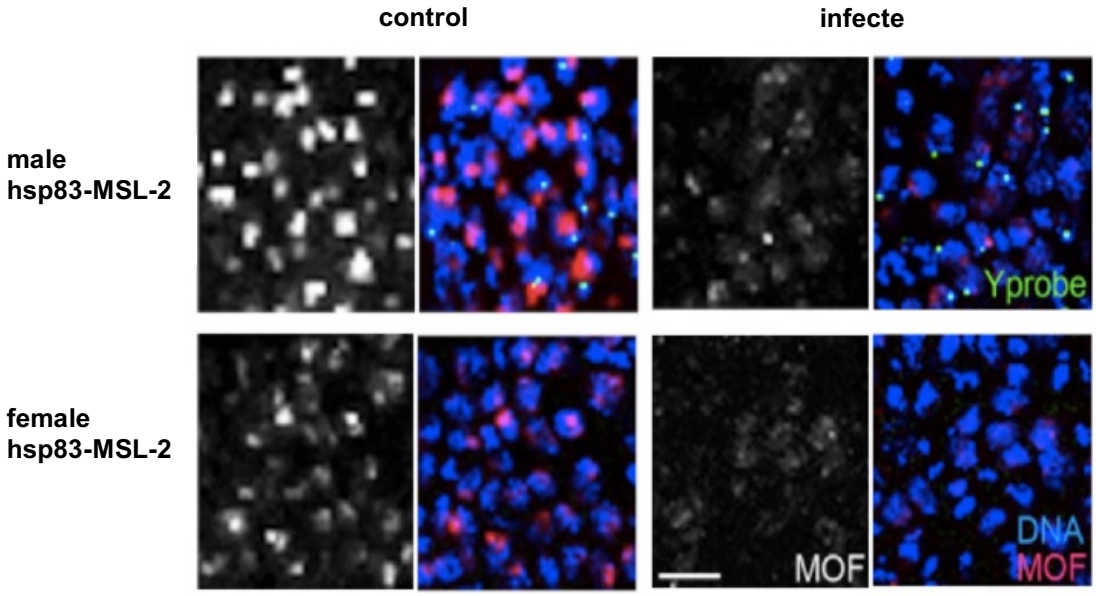



**Figure 6.** MOF is expressed weakly and dispersed throughout the nucleus in infected transgenic embryos. Scale bar: 5um.

## Discussion and Conclusions

Previous studies have shown that infection of *D. melanogaster* by the MSRO (male-killing) *Spiroplasma* strain causes male death during mid-embryogenesis. The first tissue type that appears defective during this time is the central nervous tissue, which becomes severely misshapen shortly after central nervous system (CNS) formation (Martin et al., 2013). In a separate study, researchers performed genetic experiments to show that mutational loss of any of the genes encoding a major DCC component (like the MSL proteins) results in a suppression of male embryo death due to *Spiroplasma* (Veneti et al., 2005). This finding suggests that male-killing by *Spiroplasma* needs a functional DCC.

In this thesis I performed experiments to investigate how *Spiroplasma* might require the DCC for inducing male lethality. Specifically, through my visualization of the DCC (MOF) and its acetylation activity (H4K16ac) in infected males with specific antibodies, I found that the DCC forms at its normal time (several hours after embryonic development starts) which is similar to uninfected males. However, I saw that the DCC becomes abnormally distributed across the entire nucleus in infected males, when normally the DCC localizes only to the X euchromatin. My microscopic resolution does not allow us to determine if DCC mis-localization is a random effect or if the DCC goes to certain abnormal regions on the other chromosomes, but the abnormal histone acetylation across the nucleus suggests that there would be major gene mis-expression across the genome as a result. This type of experimentation was not a part of my thesis, but our research group (mainly by postdoc John Aldrich) found evidence through analysis of RNA-Seq data that in fact many genes are either overexpressed or under-expressed in

infected males. This finding fits my observations of abnormal, widespread H4 acetylation. I can also eliminate other alternative hypotheses that we had proposed, such as that perhaps *Spiroplasma* killed males by blocking not the DCC localization but instead only the acetylation activity or caused over-acetylation activity only on the X chromosome (with no DCC mis-localization).

As a separate but related part of my thesis work, I wanted to test further if the presence of a functional DCC is in fact the direct cause of male-killing. To do this, I expressed the MSL-2 protein in *Spiroplasma* infected females using a leaky transgene. Normally, females do not produce this protein, although they do produce all other components of the DCC. As a result, the DCC does not normally form in females (Conrad and Akhtar, 2012). This could be why only males are killed by *Spiroplasma* and females are allowed to live, even though both sexes are infected. I found that uninfected females expressing low levels of MSL-2 are sick but they survive. On the other hand, *Spiroplasma* infected females expressing MSL-2 experience 50% lethality in comparison to the uninfected females. This lethality level was obtained from expressing very low levels of MSL-2. I could not increase expression of MSL-2 in order to see if the lethality level went up because high levels of MSL-2 presence in females have been found to be lethal (Kelley, 1995). I also microscopically examined the patterns of the DCC and histone acetylation in these infected DCC-expressing female embryos to see what was exactly happening to these transgenic females at a cellular level. Just like in wild type infected males, these transgenic infected females showed MOF not localized to the X chromosome, but instead localized across the nucleus. Together my experiments strongly support previous ideas that male-killing does require a functional DCC, that male-killing

involves abnormal localization of the DCC to incorrect regions of the genome, and that this is what causes abnormal histone acetylation.

My results may also provide an explanation for our lab's previous work that suggests that *Spiroplasma* causes defects in the CNS just after it forms in embryos. It is puzzling why the CNS is the first tissue to become visibly abnormal when *Spiroplasma* is known to infect many if not most of the embryo's cells and also to reside within the cell-less interior (Haselkorn, 2010). This is counterintuitive as the CNS is produced from ectoderm, the outer layer of cells of the young embryo, which should not have *Spiroplasma* present (Schmidt et al., 1997). Also, the cells of the new CNS detach from this layer of ectoderm and undertake the CNS fate, moving to specific regions on the ventral side of the embryo to form the 'ventral midline'. These early neural cells actually use signals of the ectoderm, that are a part of the axis patterning, to migrate to the proper places and divide at the appropriate times. The main cell type that I saw DCC mis-localization in were the surface cells, which are a part of the ectoderm tissue. It might be the case that as genes become mis-expressed in these cells because of *Spiroplasma* infection, they do not express the proper signals required for neural cell migration and division. The result would be the defective CNS formation that previous students Jennifer Martin and Trisha Chong observed through microscopy (Martin et al., 2013). This idea also matches the timing of the DCC mis-localization, which probably happens as the DCC forms and just several hours before the CNS defective formation occurs.

How does *Spiroplasma* actually cause DCC mis-localization? This is an important question that will require much further experimentation. It is known from previous studies that certain *Spiroplasma* strains and other symbiotic bacteria like *Wolbachia*

actually produce proteins that are secreted into the host's cells and tissues. Just recently, a group published a study showing that *Wolbachia* produces two proteins encoded by a virus that can mimic the conditional sterility caused by *Wolbachia*. I also attended the *D. melanogaster* conference recently, where a researcher in Switzerland (Bruno Lemaitre) presented preliminary work which showed that his lab had identified a viral *Spiroplamsa* protein that kills only males but not females when expressed transgenically. The question though is how such a *Spiroplasma* toxin acts to kill males. There are several interesting possible explanations. One is that *Spiroplasma*'s toxin directly binds to a component of the DCC and causes it to be 'sticky' to all regions of the genome. Another possible explanation is that this toxin does not bind to the DCC, but instead to the system that makes the DCC bind specifically to the X chromatin. The current model is that the DCC uses several different ways of localizing only to the X chromosome. One way is that the DCC forms as the roX RNA, a structural RNA that is a scaffolding component of the DCC and located on the X chromosome, is transcribed (Conrad and Akhtar, 2012). So during roX transcription, the DCC assembly happens there at the X chromosome. Another way the DCC associates only with the X chromosome is that the X chromosome has a higher number of MREs, which are MSL protein Recognition Elements that are bound at a higher affinity by DCC (Meller et al., 1997). A protein called CLAMP might be another target of this toxin because CLAMP is thought to act as a linker protein that binds to chromatin and also to the DCC. Previous research has shown that mutational loss of CLAMP results in DCC mis-localization (Soruco et al., 2013). It is possible that *Spiroplasma* could disrupt any of these factors in order to make the DCC localize to ectopic regions in the genome. Future experiments in our lab can take advantage of

mutations in these factors to test these ideas about the relationship between male-killing and the DCC.

In conclusion, my results support a model for *Spiroplasma*-caused male-killing. In this model, *Spiroplasma* secretes a toxin in both male and female embryos. This toxin binds with the DCC or with a component of the system that helps the DCC bind specifically to the X chromosome. As a result, the DCC is mis-localized to regions across the genome. This causes abnormal histone acetylation at these regions and, therefore, genome-wide gene mis-expression occurs. This mis-expression causes embryonic cells, especially those in the ectoderm, to not express the proper factors needed for their further development and normal formation of the CNS. Eventually enough abnormal development occurs to cause infected male embryos to die. The infected female embryos are not affected by *Spiroplasma* because they do not form the DCC and do not go through dosage compensation, so the toxin does not have an effect on them. Overall, my results help us to better understand how bacterial symbionts in nature can selectively kill only one sex so that they themselves can be transmitted more efficiently. In the case of *Spiroplasma*, male-killing happens through the male-specific process of dosage compensation. But in other insects, male-killing has to happen through other pathways because dosage compensation would not involve the same process as it does in *D. melanogaster*. It would be interesting to learn about the molecular interactions in these different male-killing cases as more research is performed.

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